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Autographa californica Multiple Nucleopolyhedrovirus Nucleocapsid Protein BV/ODV-C42 Mediates the Nuclear Entry of P78/83[▽]

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Autographa californica **multiple nucleopolyhedrovirus (AcMNPV)** ***BV/ODV-c42*** **(*orf101*; *c42*), which encodes a 41.5-kDa viral nucleocapsid protein with a putative nuclear localization signal (NLS) motif at the C terminus, is a highly conserved gene among members of the *Baculoviridae* family. C42 is demonstrated to be essential for AcMNPV propagation and can bind to nucleocapsid protein P78/83, a viral activator for the actin-related protein 2/3 (ARP2/3) complex to initiate nuclear actin polymerization, which is essential for viral nucleocapsid morphogenesis during AcMNPV infection. Here, we report the identification of a novel pathway through which *c42* functions in nucleocapsid assembly. Cotransfection of Sf9 cells with *c42* and *p78/83* plasmids demonstrated that C42 was capable of recruiting P78/83 to the nuclei of uninfected cells and that the NLS motif of C42 was essential for this process. To validate this nuclear relocation mode in bacmid-transfected cells, a *c42*-disrupted bacmid ($vAc^{c42ko\text{-}gfp}$) and rescued bacmids with wild-type *c42* ($vAc^{c42res\text{-}gfp}$) or with NLS coding sequence-mutated *c42* ($vAc^{c42nls\text{-}gfp}$) were prepared. By immuno-staining, P78/83 was found to be localized in the cytoplasm of either $vAc^{c42ko\text{-}gfp}$- or $vAc^{c42nls\text{-}gfp}$-transfected cells, whereas P78/83 was relocated to the nuclei of $vAc^{c42res\text{-}gfp}$-transfected cells. Furthermore, F-actin-specific staining confirmed that there was no actin polymerization activity in the nuclei of either $vAc^{c42ko\text{-}gfp}$- or $vAc^{c42nls\text{-}gfp}$-transfected cells, which might be attributed to the absence of nuclear P78/83, an activator of the ARP2/3 complex to initiate nuclear actin polymerization. We therefore hypothesize a mode of action where C42 binds to P78/83 in the cytoplasm to form a protein complex and cotransports to the nucleus under the direction of the NLS motif in C42 during AcMNPV infection.**

Baculoviruses are rod-shaped, enveloped viruses with circular double-stranded DNA genomes ranging in size from 80 kb to 180 kb (7). One of the unique features of *Baculoviridae* members is that during the viral replication cycle, they produce two distinct infectious forms: a budded-virus (BV) form comprising a single virion enveloped by a plasma membrane, which is associated with systemic infection, and an occlusion-derived virus (ODV) form comprising enveloped virions embedded within a crystalline matrix of protein, which is involved in lateral transmission between insects when released into the environment upon the death of the host. *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is one of the best-characterized baculoviruses and widely used as a biopesticide and protein expression vector (4, 11).

During the baculovirus replication cycle, not only viral gene products but also host cell proteins are recruited to assist virus propagation. One of the most striking events is the formation of F-actin cables in the cytoplasm and nucleus in a separate and sequential manner, which is essential for nucleopolyhedrovirus progeny production in insect cells (2, 10, 15). The cytoplasmic G-actin monomers begin to polymerize shortly after viral nucleocapsids are released to the cytoplasm from endosomes (2). The resulting F-actin cables are possibly involved in helping nucleocapsid penetration into the nucleus. They are formed as early as 0.5 h postinfection (hpi), and this cytoplasmic actin polymerization process appears to be independent of viral protein synthesis (2). The nuclear actin polymerization takes place within 2.0 ± 0.4 h after cytoplasmic G-actin monomers are driven to accumulate in the nucleus by six viral early gene products, including IE-1, PE38, HE65, Ac004, Ac102, and Ac152 (6, 14). The relocated G-actin monomers are attached to the host cell actin-related protein 2/3 (ARP2/3) complex, activated by the viral WASP homologous protein P78/83 (*orf9*) to initiate the polymerization process in the nuclei of infected cells, which leads to the formation of nuclear F-actin cables that are essential for viral nucleocapsid assembly and morphogenesis (6, 15, 23). Therefore, the prerequisites for virus-induced nuclear actin polymerization are dependent on the accumulation of at least three elements in the nucleus: G-actin, P78/83, and the ARP2/3 complex. However, both P78/83 and the ARP2/3 complex have been shown to be self-localized in the cytoplasm of uninfected cells (5). Little is known about how these two molecules are recruited to the nucleus during baculovirus infection.

BV/ODV-c42 (*orf101*; *c42*) is a highly conserved, viral late gene which encodes a 42-kDa viral nucleocapsid protein (1). A putative nuclear localization signal (NLS) (^{357}KRKK^{360}) motif was found at the C terminus of C42 and is thought to be associated with C42's subcellular distribution pattern in AcMNPV-

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infected cells (1). Deletion analysis demonstrated that *c42* is an essential gene for nucleocapsid assembly, whereas no interruption in viral genomic DNA replication was observed (20). Previous reports also provide evidence that C42 binds to P78/83 in a nucleocapsid-independent manner in infected cells (1). Therefore, we hypothesize that one of the pathways through which C42 participates in BV propagation is via interaction with P78/83.

In this study, we have explored the relationship between C42 and P78/83, in particular, the nuclear entry mechanisms of these two molecules in insect cells. We identified that C42 was capable of recruiting P78/83 to the nuclei of either uninfected cells or bacmid-transfected cells and that the NLS motif in C42 was not only a *cis*-acting element that determines C42's nuclear self-localization, but also a *trans*-acting factor that controls P78/83's nuclear relocation. Moreover, no nuclear actin polymerization activities were observed in the cells with transfection of either a *c42*-disrupted bacmid or a rescued bacmid with the NLS coding sequence-mutated *c42*, probably as a consequence of the absence of P78/83 from the nuclei, which resulted in an inability to activate the ARP2/3 complex.

MATERIALS AND METHODS

Cell culture and virus. *Spodoptera frugiperda* IPLB-Sf21-AE colonial isolate 9 (Sf9) cells were cultured at 27°C in Grace's medium (Invitrogen) with a supplement of 10% fetal bovine serum (FBS) (Invitrogen) (21). The AcMNPV genome (strain E2) was used as a template for the cloning of viral genes, and AcMNPV recombinant bacmids were derived from bacmid bMON14272 (Invitrogen) and propagated in *Escherichia coli* strain DH10B (12).

Preparation of antibodies. The coding sequences of *c42* (full length) and *p78/83* (residues 321 to 407) were amplified by PCR with primer sets C42-F/R and P78/83-961/1247 (Table 1), respectively, and cloned into pMal-c2x (New England BioLabs). The resulting constructs, pMal-c42 and pMal-p78/83, were transformed into TB-1 competent *E. coli* cells, and the MBP fusion proteins were induced by isopropyl-β-D-thiogalactopyranoside. After affinity purification by amylose resin (New England Biolabs), the MBP-fused viral proteins were used to immunize rabbits. To remove nonspecific antibodies from the collected sera, 1,000 ml of TB-1 cells harboring pMal-c2x were induced by isopropyl-β-D-thiogalactopyranoside, redissolved in 100 ml of phosphate-buffered saline (PBS), and thoroughly sonicated with a VCX500 ultrasonic processor (Sonics & Materials). The debris of *E. coli* cells were centrifuged at 18,000 × *g* for 20 min, and the sediments containing insoluble bacterial proteins and MBP inclusion bodies were redissolved in 50 ml of PBS. The resulting lysates were then used to neutralize the sera in a 1:1 volume ratio. After being gently shaken at 37°C for 4 h, the serum-lysate mixtures were centrifuged at 20,800 × *g* for 30 min to separate the immunoprecipitates, and the supernatants were collected and stored at −80°C. When the antibodies were about to be used for immunostaining, 500 μl of freshly sonicated cell extracts from 1×10^7 uninfected Sf9 cells were used to neutralize the sera (5 μl) again in a 100:1 volume ratio. After incubation and centrifugation processes similar to those described above in this section, the supernatants containing specific antibodies were collected and subjected to immuno-staining as primary antibodies. The specificity of the antibodies was tested by Western blotting and immuno-staining with the indicated control samples.

Cotransfection assays. Full-length coding sequences of wild-type *c42*, NLS coding sequence-mutated *c42* (^{357}KRKK^{360} mutated to ^{357}AAAA^{360}), and wild-type *p78/83* were amplified by PCR with primer sets C42-F/R, C42-F/NLS, and P78/83-F/R (Table 1), respectively. The resulting fragments were inserted into pIZ/V5-His (Invitrogen) to generate transient expression vectors pIZ-c42^{wt}, pIZ-c42^{nls}, and pIZ-p78/83. For the labeling of C42 by fusion with EGFP, an *egfp* gene amplified from pEGFP-N1 (Clontech) with primer set EGFP-F/R (Table 1) was cloned in frame into pIZ-c42^{wt}, pIZ-c42^{nls}, and pIZ/V5-His between the HindIII and KpnI sites to generate pIZ-gfp-c42^{wt}, pIZ-gfp-c42^{nls}, and pIZ-gfp, respectively. For cotransfection assays, Sf9 cells (1×10^6) on coverslips were transfected with pIZ-p78/83 plus pIZ-gfp (1 μg each), pIZ-p78/83 plus pIZ-gfp-c42^{wt} (1 μg each), pIZ-p78/83 plus pIZ-gfp-c42^{nls} (1 μg each), or pIZ-gfp (2 μg) by Cellfectin (Invitrogen) according to the manufacturer's protocol. At 24 h posttransfection (hpt), the cells were immuno-stained by anti-P78/83 antibody and viewed with a confocal microscope. (For details see the section below describing bacmid transfection and confocal microscopy).

TABLE 1. The primer list

Name	Sequence
C42-F	CGCGGATCCACGATGAGCGCTATCGCGT
C42-R	CCCTCTAGATTAATATTTTTTACGCTTTGCATTC
C42-NLS	TGCTCTAGATTAATATGCTGCGGCCGCTGCATTCGACGACTGAACTC
C42ko-DN	CAAGTGATAGTTACAGAGTAAGTGTGCGTCGTCGGTCACGCATATGAATATCCTCCTTA
C42ko-UP	AAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTGTAGGCTGGAGCTGCTT
C42-P	CGCGGATCCAACTTCATGATAGAAATGGGCCG
CAT-366	CACGCCACATCTTGCGAATA
CAT-500	TGGCAATGAAAGACGGTGAG
UP-500	TGACAACATTCTTTACAAGTATTCCC
DN-500	GATATTTGTAACGTGCTAAT
P78/83-F	CCGGAATTCCATGACGAATCGTAGATATGAAT
P78/83-R	TGCTCTAGATTAAGCGCTAGATTCTGTGCG
P78/83-961	CGCGGATCCCCATCGCTTTCTAACGTGTTGT
P78/83-1247	TGACTCGACGTAAACACGTTAAATA
EGFP-F	CCCAAGCTTCGCCACCATGGTGAGCAA
EGFP-R	CGGGGTACCCTTGTACAGCTCGTCCATGC
Q-65972F	CGTAGTGGTAGTAATCGCCGC
Q-66072R	AGTCGAGTCGCGTCGCTTT
Q-IE2-F	CAGAACCCGAAGAAGAAGTAGAGG
Q-IE2-R	CGAGTGACGTTAATGGGCATA
Q- P78/83-F	CCTTCTCCCGTGCCTAACATTCCAG
Q- P78/83-R	TGAGGTTCCGTCGGCATTGGTG
Q-P10-F	TTTGGACGGTTTGCC
Q-P10-R	TTGGAACTGCGTTTACC
Q-18s-F	TCTTTCAAATGTCTGCCTTATCAAC
Q-18s-R	GGATGTGGTAGCCGTTTCTCA

Gene disruption. To disrupt *c42* in the AcMNPV genome, bacmid bMON14272 served as a target for gene disruption and λ Red recombination was employed according to the modified method of Datsenko and Wanner (5). Briefly, a chloramphenicol resistance gene (chloramphenicol acetyltransferase or *cat*) cassette was amplified from a pKD3 template plasmid by PCR with primer set C42ko-DN/UP (Table 1). Each contained a 40-nucleotide (nt)-long homologous arm at the 5′ end in accordance with either C42ko-DN (nt 88006 to 88046) or C42ko-UP (nt 87175 to 87215) of the AcMNPV genome (GenBank accession number NC_001623.1). The gel-purified PCR product was digested by 20 units of DpnI (New England Biolabs) overnight to completely remove any remaining template plasmids before it was electro-transformed into arabinose-preinduced DH10B competent *E. coli* cells harboring bMON14272 and λ Red recombinase-encoding plasmid pKD46. The resulting transformed DH10B cells were incubated at 37°C for 4 h in SOC (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) medium with gentle shaking before being spread onto an LB agar plate containing kanamycin and chloramphenicol. The colonies resistant to both antibiotics were selected after 48 h of incubation at 37°C, and the bacmid constructs were extracted for further screening. Two sets of primers, UP-500/CAT-500 and DN-500/CAT-366 (Table 1), were used to confirm the proper replacement of *c42* with the *cat* cassette by PCR. To ensure that there was no additional copy of the *cat* cassette randomly inserted into bMON14272 except at the *c42* N-terminal coding region, quantitative reverse transcription-PCR (qRT-PCR) was performed in the knockout candidates to compare the copy number of *cat* with that of *p78/83*, a single-copy gene in the AcMNPV genome without an EcoRI recognition site. About 0.01 μg of the knockout candidate and the quantification standard bacmid vAc$^{38k\text{-}ko}$ (a kind gift from Kai Yang, Sun Yat-sen University) (24) was digested by EcoRI, split into 1-μl aliquots, and submitted to Sybr green I real-time PCR master mix

(Toyobo) as a template, with primer sets CAT-366/500 (for the evaluation of *cat*) and Q-P78/83-F/R (for the evaluation of *p78/83*) (Table 1). qRT-PCR was performed in triplicate with the Opticon continuous fluorescence detection system (MJ Research) by using the following conditions: hot-start DNA polymerase activation at 95°C for 1 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 15 s, and extension at 72°C for 15 s. The successful knockout bacmid was termed vAc^{c42ko}.

Construction of donor plasmids and transposition of bacmid constructs. A series of donor vectors was constructed based on plasmid pFbdg, which was modified from pFastBac Dual (Invitrogen) with an *egfp* gene driven by a *p10* promoter to serve as an indicator for positive bacmid transfection or virus infection. pFbdg was transposed to vAc^{c42ko} by the Bac-to-Bac system (Invitrogen) to generate $vAc^{c42ko\text{-}gfp}$ according to the manufacturer's protocol (12). Meanwhile, gene expression cassettes of wild-type *c42* and NLS coding sequence-mutated *c42* were amplified with primer sets C42-P/R and C42-P/NLS (Table 1), respectively, by PCR. The resulting fragments were cloned into pFbdg between the Bst1107I and XbaI sites to generate rescued bacmid $vAc^{c42res\text{-}gfp}$ and mutated bacmid $vAc^{c42nls\text{-}gfp}$ after the donor vectors were transposed to vAc^{c42ko}. All of these bacmid constructs were confirmed by PCR with primer set M13F/R (Table 1) according to the manufacturer's protocol. Correctly transposed bacmid constructs were retransformed into DH10B *E. coli* cells and screened for tetracycline sensitivity to obtain purified bacmids without helper plasmid pMON7124.

Viral propagation assays. The bacmid constructs of $vAc^{c42ko\text{-}gfp}$, $vAc^{c42nls\text{-}gfp}$, $vAc^{c42res\text{-}gfp}$, or vAc^{gfp} (wild-type control bacmid with a transposed *egfp* gene driven by the *p10* promoter [unpublished work]) were transfected to Sf9 cells, by Cellfectin (Invitrogen) according to the manufacturer's protocol. At 144 hpt, the supernatants were collected and filtered with 0.45-μm-diameter syringe filters (Sartorius) to remove cell debris before being added to uninfected Sf9 cells to initiate secondary infection. After 1 h of infection, the supernatants were discarded and the cells were replenished with 2 ml of Grace's medium with a supplement of 10% FBS and incubated at 27°C for another 144 h. Dynamic changes of the transfected cells were monitored by an Olympus IX51 inverted microscope. To evaluate the growth kinetics of viruses derived from these bacmid constructs, Sf9 cells plated at 2.5×10^5 per well on a 24-well plate were infected with BV stocks ($vAc^{c42res\text{-}gfp}$ and vAc^{gfp}) at a multiplicity of infection (MOI) of 5 or 1,000-μl supernatants from bacmid-transfected cells ($vAc^{c42ko\text{-}gfp}$ and $vAc^{c42nls\text{-}gfp}$) at 144 hpt in triplicate. Viral supernatants from 0, 12, 24, 48, 72, 96, and 120 hpi were collected, and the titers were determined by a 50% tissue culture infective dose endpoint dilution assay with EGFP expression as an indicator of positive infection.

Assays of viral gene transcription by qRT-PCR. To investigate whether the expression patterns of viral genes were altered at the transcriptional level after *c42* was disrupted from the viral genome, Sf9 cells plated at 2.5×10^5 per well on a 24-well plate were transfected with 2 μg of vAc^{c42ko} or *gp64*-KO (a kind gift from G. F. Rohrmann, Oregon State University) (19). Total RNAs were isolated by TRIzol reagent (Invitrogen) at 0, 12, 24, 48, 72, 96, and 120 hpt according to the manufacturer's protocol and dissolved in 20 μl of RNase-free water. Each sample was then treated with 1 unit of RQ1 RNase-free DNase (Promega) for 30 min before 4 μg of DNA-free RNAs from each sample were collected and split into two 2-μg aliquots; one aliquot was reverse transcribed with 1 μg of random primer (Genscript) by M-MLV (Promega), and one aliquot was evaluated by qRT-PCR with primer set Q-65972F/66072R (Table 1) (18) to determine the level of contaminated bacmid DNA present as a background. Three representative genes, *ie-2*, *p78/83*, and *p10* were chosen to investigate the transcriptional level of viral early, late, and very late genes by qRT-PCR in triplicate, and host cell 18s rRNA served as an endogenous reference. The qRT-PCR assays were performed as described above for the comparison of *cat* with *p78/83*.

Bacmid transfection and confocal microscopy. Sf9 cells grown on coverslips were transfected with bacmid constructs of $vAc^{c42res\text{-}gfp}$, $vAc^{c42ko\text{-}gfp}$, $vAc^{c42nls\text{-}gfp}$, or $vAc^{p78/83ko\text{-}gfp}$ (*p78/83*-disrupted bacmid with a transposed *egfp* gene driven by the *p10* promoter [unpublished work]) and incubated for the indicated time. After being rinsed twice with PBS, the transfected cells were subjected to sequential treatments with fixation in 3.7% paraformaldehyde, permeabilization by 100% methanol or 0.1% Triton X-100, and blocking with 3% bovine serum albumin. For immuno-staining, the cells on coverslips were incubated overnight at 4°C with preneutralized sera containing the indicated primary antibodies and 0.1% Triton X-100. After a triplicate PBS washing process, the cells were incubated at 37°C for 1 h with rhodamine-conjugated secondary antibody (PtgLab). For F-actin-specific staining, rhodamine-conjugated phalloidin (Molecular Probes) was used to label the polymerized actin in Sf9 cells by incubation with cells on coverslips for 20 min at room temperature. To distinguish the nucleus from the cytoplasm, all the samples were finally stained with Hoechst 33258 (Beyotime) before being viewed with a Leica SP2 confocal laser scanning microscope.

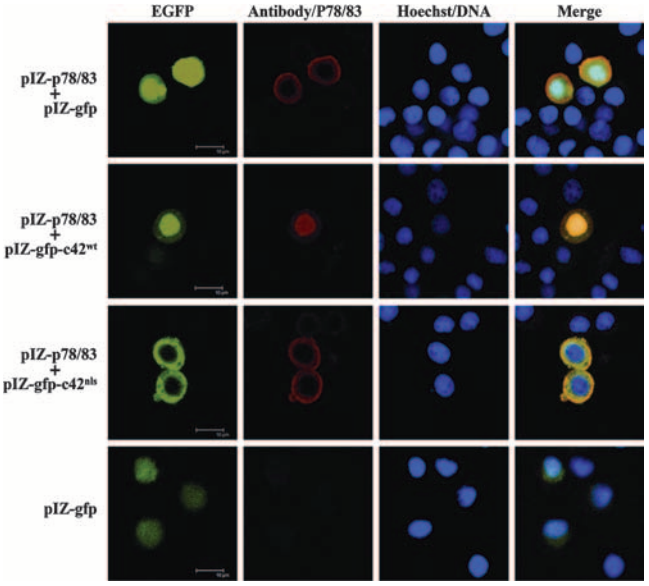

FIG. 1. Subcellular distribution patterns of P78/83 and EGFP-fused C42 in plasmid-transfected cells. pIZ-p78/83 was cotransfected to Sf9 cells with pIZ-gfp, pIZ-gfp-$c42^{wt}$, or pIZ-gfp-$c42^{nls}$. At 24 hpt, the transfected cells were fixed and permeabilized. P78/83 was immuno-stained by anti-P78/83 antibody as primary antibody and rhodamine-conjugated antibody as secondary antibody. The cellular DNA in the nuclei was stained by Hoechst 33258. The transfection of pIZ-gfp served as a control. The plasmids transfected are indicated on the left. The bar represents 10 μm.

RESULTS

Subcellular distribution patterns of C42 and P78/83 in uninfected cells. It has been demonstrated that C42 and P78/83 interact directly by the yeast two-hybrid assay and blue native electrophoresis (1). Here we further analyzed the subcellular localization of P78/83 and C42 in Sf9 cells transfected with pIZ-p78/83 and/or C42. In the Sf9 cells transfected with pIZ-p78/83 plus pIZ-gfp, the immuno-staining results showed that P78/83 was localized in the cytoplasm (Fig. 1), which was consistent with a previous report that P78/83 was self-localized in the cytoplasm of TN-368 cells (6). EGFP-fused C42 was distributed in the nucleus of the Sf9 cells transfected with pIZ-gfp-$c42^{wt}$ (data not shown). When pIZ-p78/83 was cotransfected with pIZ-gfp-$c42^{wt}$, P78/83 and C42 were colocalized in the nucleus (Fig. 1). These spatial phenotypes indicated that wild-type C42 was a nuclear self-localization protein and capable of mediating the nuclear translocation of P78/83 in uninfected Sf9 cells. To further elucidate the mechanism behind C42's property of nuclear self-localization and capability of recruiting P78/83 to the nucleus, pIZ-p78/83 was cotransfected with pIZ-gfp-$c42^{nls}$. At 24 hpt, both C42 NLS mutants and P78/83 were restricted to the cytoplasm. As a negative control, no significant rhodamine fluorescence could be observed in pIZ-gfp-transfected cells (Fig. 1). Therefore, the putative NLS motif in C42 was not only involved in mediating the nuclear entry of C42 in *cis* but essential to C42's capability to recruit P78/83 to the nuclei in *trans* in uninfected Sf9 cells.

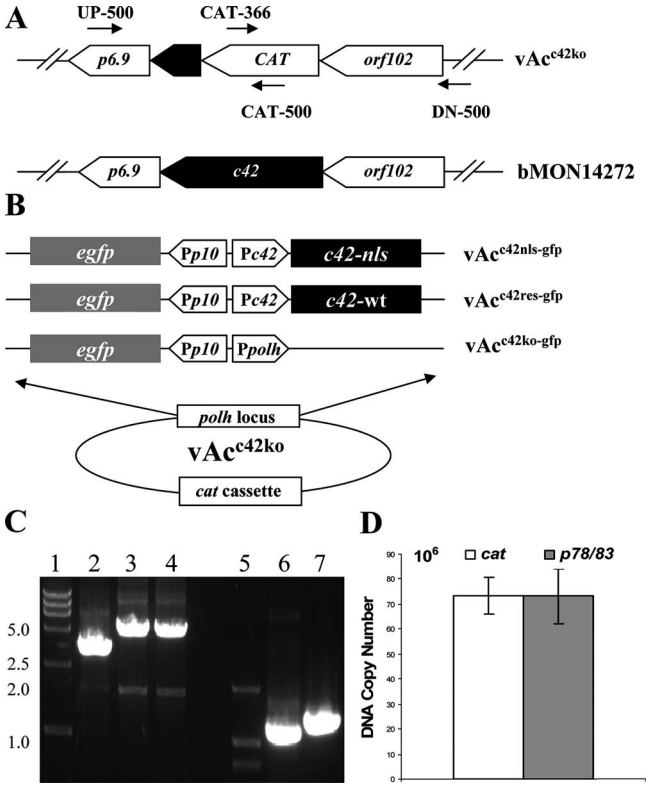

FIG. 2. Generation of recombinant AcMNPV bacmids. (A) Diagram of *c42* knockout. The N-terminal coding region (1 to 792 bp) of *c42* on AcMNPV bacmid bMON14272 was replaced with a *cat* expression cassette. The knockout bacmid was termed vAc^{c42ko}. Two sets of primers, UP-500/CAT-500 and CAT-366/DN-500 (Table 1), that were used for PCR confirmation are labeled with arrows at their corresponding loci. (B) The construction diagram of a series of transposed bacmids derived from vAc^{c42ko}. All the transposed constructs possess an *egfp* gene under the control of the *p10* promoter (P*p10*). vAc$^{c42res-gfp}$ contains a wild-type *c42* expression cassette, whereas vAc$^{c42nls-gfp}$ contains a copy of the *c42* expression cassette with a C42 NLS coding sequence mutation. Both expression cassettes are driven by *c42*'s native promoter (P*c42*). (C) Ethidium bromide-stained agarose gel of PCR amplicons from transposed and knockout candidates. Lane 1, the DNA marker DL15.000 (Takara); lanes 2 to 4, PCR amplicons of vAc$^{c42ko-gfp}$, vAc$^{c42res-gfp}$, and vAc$^{c42nls-gfp}$, respectively, using primer set M13-F/R (Table 1); lane 5, the DNA marker DL2.000 (Takara); lanes 7 and 8, the PCR amplicon of the *c42* knockout candidate using primer sets UP-500/CAT-500 and CAT-366/DN-500 (Table 1), respectively. (D) Comparison of the gene copy number of *cat* with the single-copy gene *p78/83* of the *c42* knockout candidate. Values are displayed as averages of triplicate bacmid mini-prep experiments, with error bars indicating standard deviations.

Generation of recombinant AcMNPV bacmids. To further analyze the function of the interaction between C42 and P78/83 during AcMNPV infection, a *c42*-disrupted bacmid was generated by λ Red recombination. In all the knockout candidates, nt 87215 to 88006 of the AcMNPV genome, which covered the N-terminal coding region of *c42*, were successfully replaced by the *cat* cassette (Fig. 2A), as all the PCR amplicons from UP-500/CAT-500 were about 1.3 kb (expected size was 1,313 bp) and amplicons from DN-500/CAT-366 were nearly 1.1 kb (expected size was 1,109 bp) (Fig. 2C). Furthermore, a qRT-PCR-based screening procedure confirmed that there were no additional copies of the *cat* cassette randomly inserted into the bacmid except at the target locus, as the copy number of *cat* was substantially equal to that of the single-copy gene *p78/83* (Fig. 2D). Here, vAc^{38k-ko} was chosen as a quantification standard, because it contains a single copy of *cat* in the bacmid as well as *p78/83* (24).

The *egfp*-tagged versions of the *c42* knockout, rescued, and mutated bacmid constructs were generated by the transposition of corresponding donor plasmids into the *polyhedrin* (*polh*) locus of vAc^{c42ko} (Fig. 2B). In all the transposed bacmid constructs, the *egfp* gene is controlled by a *p10* promoter. In the rescued bacmid vAc$^{c42res-gfp}$, a wild-type *c42* expression cassette is controlled by its native promoter (nt 88005 to 88731 of the AcMNPV genome), while in the mutated bacmid vAc$^{c42nls-gfp}$, the NLS coding sequence, ^{357}KRKK^{360}, in the *c42* expression cassette was mutated to ^{357}AAAA^{360}. The transposed bacmid constructs were confirmed by PCR with M13-F/R. The amplicons from vAc$^{c42ko-gfp}$, vAc$^{c42res-gfp}$, and vAc$^{c42nls-gfp}$ were about 3.2, 5, and 5 kb, respectively (Fig. 2C), which is consistent with the predicted sizes, indicating that the constructs are correct.

Virus propagation assays. The bacmid constructs of vAc$^{c42ko-gfp}$, vAc$^{c42nls-gfp}$, vAc$^{c42res-gfp}$, and vAc^{gfp} were separately transfected into Sf9 cells. The EGFP fluorescence from all transfected cells became visible as early as 36 hpt and gradually became stronger as the incubation time increased. Unlike the ubiquitous EGFP fluorescence in vAc$^{c42res-gfp}$- or vAc^{gfp}-transfected cells, only isolated fluorescent cells were observed in the transfected cells of either vAc$^{c42ko-gfp}$ or vAc$^{c42nls-gfp}$, even at 144 hpt (Fig. 3A). These EGFP expression phenotypes strongly indicated that vAc$^{c42ko-gfp}$ and vAc$^{c42nls-gfp}$ failed to produce infectious BV particles when transfected to Sf9 cells, while the transcription and translation capability of the viral very late gene remained uninterrupted, as the *egfp* gene driven by the *p10* promoter successfully expressed EGFP. Secondary virus infection tests further proved the hypothesis that both C42 and its NLS motif were essential to BV propagation, as no EGFP fluorescence was observed 6 days after the supernatants from vAc$^{c42ko-gfp}$- or vAc$^{c42nls-gfp}$-transfected cells (144 hpt) were added to uninfected cells to initiate a secondary infection (Fig. 3A). As expected, when supernatants from either vAc$^{c42res-gfp}$- or vAc^{gfp}-transfected cells (144 hpt) were added to uninfected cells, EGFP fluorescence became widely spread within 36 hpi (Fig. 3A). To quantify the virus production capability of these bacmid constructs, virus growth kinetics assays were performed. For these experiments, Sf9 cells were infected with either viral stocks (vAc$^{c42res-gfp}$ and vAc^{gfp}) at an MOI of 5 or viral supernatants (vAc$^{c42ko-gfp}$ and vAc$^{c42nls-gfp}$) from bacmid-transfected cells at 144 hpt, and the BV titers from virus-infected cells were determined by a 50% tissue culture infective dose endpoint dilution assay at selected time points. In accordance with the preliminary results obtained from bacmid transfection-infection assays, vAc$^{c42res-gfp}$ and vAc^{gfp} shared similar kinetics in virus production, whereas no BV titers could be detected from vAc$^{c42ko-gfp}$- or vAc$^{c42nls-gfp}$-infected cells at any of the selected time points (Fig. 3B), indicating that the restoration of wild-type *c42* to the *polh* locus successfully rescued the BV production capability to the normal level and that the knockout of *c42* did not interrupt the regulatory sequences of surrounding genes.

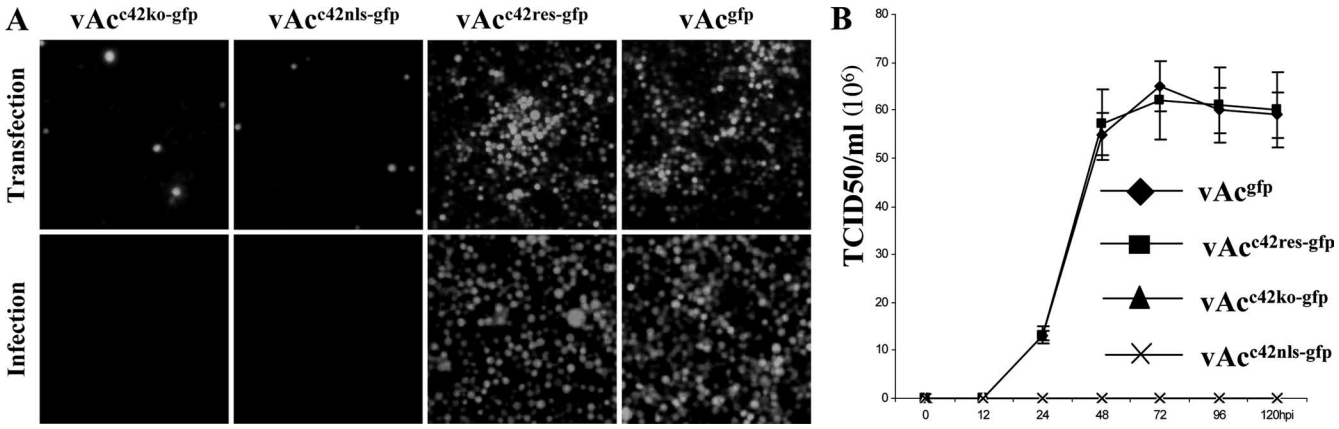

FIG. 3. Viral propagation assays. (A) Analysis of viral propagation capability in Sf9 cells by transfection and secondary infection. The bacmids shown in the top row were used to transfect Sf9 cells. At 144 hpt, the cells were visualized and the supernatants were collected to initiate secondary infection. (B) Virus growth curve of knockout, rescued, mutated, and wild-type bacmids. BV stocks ($vAc^{c42res\text{-}gfp}$ and vAc^{gfp}; MOI of 5) or viral supernatants ($vAc^{c42ko\text{-}gfp}$ and $vAc^{c42nls\text{-}gfp}$; 144 hpt) were used to infect Sf9 cells. The viral titers were determined by monitoring EGFP expression at the indicated times. The data points indicate the averages of triplicate transfections, and the error bars represent standard deviations. TCID50, 50% tissue culture infective dose.

Transcription patterns of viral genes in bacmid-transfected cells. A previous report demonstrated that the knockout of *c42* did not alter the viral DNA replication pattern (20). To gain a more comprehensive understanding of how *c42* participates in AcMNPV propagation, qRT-PCR assays were performed to investigate whether the knockout of *c42* would alter the expression patterns of viral genes at the transcriptional level. Here, *gp64*-KO was chosen as a wild-type control because this mutant bacmid loses the capability to initiate cell-to-cell infection due to the knockout of *gp64*, which is an essential gene required for viral nucleocapsids to egress from infected cells, while other propagation processes like viral gene transcription should be unaffected (13, 17, 19). The qRT-PCR results demonstrated that the transcripts of all the three representative genes in vAc^{c42ko}-transfected cells continued to accumulate at rates similar to those of their counterparts in *gp64*-KO-transfected cells from 24 to 96 hpt (data not shown). Moreover, qRT-PCR measurement of DNase-treated RNA proved that bacmid DNA was undetectable in all transfection samples (data not shown). Therefore, the quantitative patterns of the three viral transcripts in vAc^{c42ko}-transfected cells were consistent with those of their counterparts in *gp64*-KO-transfected cells, suggesting that the knockout of *c42* did not interrupt the expression patterns of early, late, and very late viral genes, including *p78/83* at the transcriptional level in bacmid-transfected cells.

Subcellular distribution patterns of C42 and P78/83 in bacmid-transfected cells. Cotransfection assays demonstrated that the nucleus-localized protein C42 was capable of recruiting P78/83 to the nuclei and the putative NLS motif in C42 was an essential structure for the mediation of the nuclear entry of both C42 and P78/83 in uninfected cells. To validate these subcellular distribution patterns and nuclear entry mechanisms in bacmid-transfected cells, immuno-staining assays were performed on Sf9 cells with transfection of the indicated bacmid constructs. To investigate the subcellular distribution pattern of C42, anti-C42 antibody was used to trace C42 in $vAc^{c42res\text{-}gfp}$-, $vAc^{c42nls\text{-}gfp}$-, or $vAc^{c42ko\text{-}gfp}$-transfected cells. At 24 hpt, C42 in $vAc^{c42res\text{-}gfp}$-transfected cells was found to be localized predominantly in the nuclei (1), while a strong rhodamine fluorescence signal of C42 was visible throughout the cytoplasm in $vAc^{c42nls\text{-}gfp}$-transfected cells (Fig. 4A). As a negative control, no significant rhodamine fluorescence could be observed in $vAc^{c42ko\text{-}gfp}$-transfected cells (Fig. 4A). These spatial phenotypes indicated that the putative NLS motif was an essential structure for the nuclear entry of C42 either in uninfected cells or in bacmid-transfected cells and that nucleus-localized C42 was essential for BV propagation as $vAc^{c42nls\text{-}gfp}$ lost its capacity to generate infectious viral particles when transfected into Sf9 cells (Fig. 3D). To elucidate the relationship between C42 and the subcellular distribution pattern of P78/83 in bacmid-transfected cells, anti-P78/83 antibody was used to trace P78/83 in $vAc^{c42res\text{-}gfp}$-, $vAc^{c42ko\text{-}gfp}$-, $vAc^{c42nls\text{-}gfp}$-, or $vAc^{p78/83ko\text{-}gfp}$-transfected cells. At 24 hpt, P78/83 was found to be localized in the cytoplasm in both $vAc^{c42ko\text{-}gfp}$- and $vAc^{c42nls\text{-}gfp}$-transfected cells, whereas it was relocated to the nuclei in $vAc^{c42res\text{-}gfp}$-transfected cells (Fig. 4B). Regarding the negative control, no significant rhodamine fluorescence could be observed in $vAc^{p78/83ko\text{-}gfp}$-transfected cells (Fig. 4B). These spatial phenotypes not only were consistent with previous findings that C42 and its NLS motif were essential for the nuclear relocation of P78/83 but also extended this relocation mode from uninfected cells to bacmid-transfected cells, suggesting that one of the pathways through which C42 participates in BV propagation is by mediating the nuclear relocation of P78/83, which is an essential protein for virus-induced nuclear actin polymerization and nucleocapsid morphogenesis (6).

Actin polymerization activities in the nuclei of bacmid-transfected cells. Via its role as a viral activator of the nuclear ARP2/3 complex to initiate the actin polymerization process, P78/83 was shown to be localized in the cytoplasm without help from wild-type C42 (Fig. 4B). It was therefore of interest to investigate the actin polymerization activities in the nuclei of either $vAc^{c42ko\text{-}gfp}$- or $vAc^{c42nls\text{-}gfp}$-transfected cells. Here, rhodamine-conjugated phalloidin was used to stain the F-actin cables specifically, and all the samples were viewed at 36 hpt to

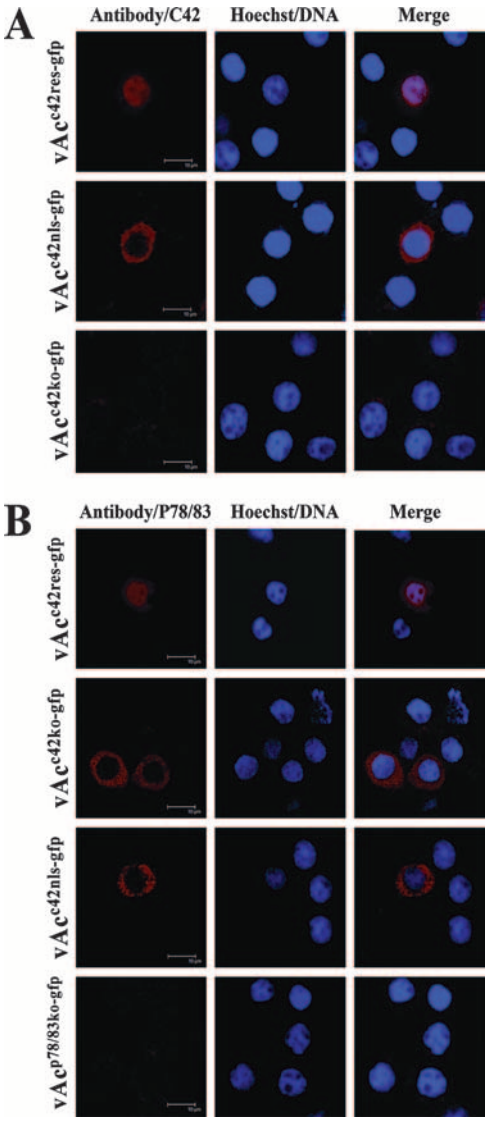

FIG. 4. Subcellular distribution patterns of C42 and P78/83 in bacmid-transfected cells. (A) The bacmid constructs of $vAc^{c42res\text{-}gfp}$, $vAc^{c42nls\text{-}gfp}$, and $vAc^{c42ko\text{-}gfp}$ were transfected to Sf9 cells. At 24 hpt, C42 was immuno-stained by anti-C42 antibody as primary antibody and rhodamine-conjugated antibody as secondary antibody. (B) The bacmid constructs of $vAc^{c42res\text{-}gfp}$, $vAc^{c42ko\text{-}gfp}$, $vAc^{c42nls\text{-}gfp}$, and $vAc^{p78/83ko\text{-}gfp}$ were transfected to Sf9 cells. At 24 hpt, the P78/83 was immuno-stained by anti-P78/83 antibody as primary antibody and rhodamine-conjugated antibody as secondary antibody. The cellular DNA in the nuclei was stained by Hoechst 33258. The bacmids transfected are indicated on the left, and the methods of detection are indicated on the top. The bar represents 10 μm.

ensure that there were visible fluorescent signals that could be used to distinguish the positive transfected cells with EGFP expression from the negative transfected cells without EGFP expression. In contrast to the $vAc^{c42res\text{-}gfp}$-positive transfected cells in which significant F-actin fluorescence was found in the nuclear region, neither $vAc^{c42ko\text{-}gfp}$- nor $vAc^{c42nls\text{-}gfp}$-positive transfected cells displayed visible nuclear F-actin fluorescence (Fig. 5), indicating that there were no actin polymerization activities in the nuclei. As a negative control, the nuclear actin

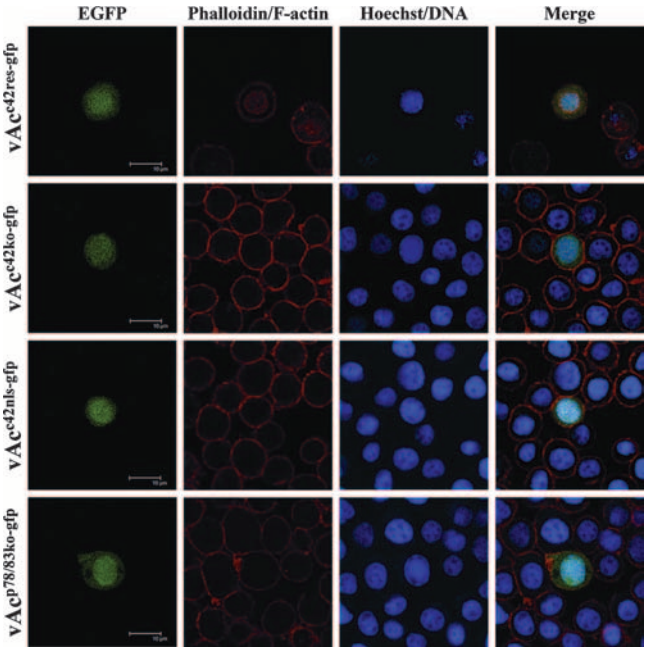

FIG. 5. Nuclear actin polymerization status in bacmid-transfected cells. The bacmids of $vAc^{c42res\text{-}gfp}$, $vAc^{c42ko\text{-}gfp}$, $vAc^{c42nls\text{-}gfp}$, and $vAc^{p78/83ko\text{-}gfp}$ were transfected to Sf9 cells. At 36 hpt, the transfected cells were fixed and permeabilized. The actin filaments were stained by F-actin-specific rhodamine-phalloidin (Molecular Probes), the cellular DNA in the nuclei was stained by Hoechst 33258, and the EGFP fluorescence was directly observed under a confocal microscope. The bacmids transfected are indicated on the left, and the methods of detection are indicated on the top. The bar represents 10 μm.

polymerization activity in $vAc^{p78/83ko\text{-}gfp}$-positive transfected cells shared the same phenotype with either $vAc^{c42ko\text{-}gfp}$- or $vAc^{c42nls\text{-}gfp}$-transfected cells generated from the knockout of *p78/83* from the viral genome (Fig. 5). As qRT-PCR results had demonstrated that the knockout of *c42* did not interrupt the expression patterns of viral genes, including *p78/83*, one of the possible reasons for nuclear actin polymerization deficiency in either $vAc^{c42ko\text{-}gfp}$- or $vAc^{c42nls\text{-}gfp}$-transfected cells was the absence of nucleus-localized P78/83, which led to the ARP2/3 complex remaining in an inactivated state and unable to initiate the actin polymerization process in the nuclei of bacmid-transfected cells.

DISCUSSION

The replication cycle of AcMNPV in Sf9 cells is always accompanied by the recruitment of various viral or cellular proteins to the nucleus to assist in viral propagation processes, such as viral DNA replication, gene transcription, or nucleocapsid assembly. Certain viral proteins, such as IE-1 and LEF-3, are capable of self-localization to the nuclei in either uninfected cells or infected cells (3, 16), whereas other kinds of viral proteins, like P143 and P78/83, are self-localized to the cytoplasm in uninfected cells but relocated to the nuclei of infected cells (3, 6). Here, we have demonstrated that C42 is a nuclear self-localization protein and capable of recruiting P78/83 to the nuclei of Sf9 cells.

Homologs of *c42* are found in almost all the sequenced

genomes of *Baculoviridae* family members. Multiple sequence comparison showed that the NLS motif in C42, which belongs to the pat4 type, is one of the most conserved sequences in all the homologs of C42 either in group I or group II nucleopolyhedrovirus (1, 8). Although the molecular mass of C42 (approximately 41.5 kDa) is very close to the 40-kDa limit, which is the empirical threshold for energy-free diffusion through the nuclear pore (8), the nuclear distribution pattern of C42 is absolutely dependent on the NLS motif at its C terminus. The mutation of this motif will change the subcellular distribution pattern of C42 from the nuclei to the cytoplasm in either uninfected cells or infected cells, which was proved by either EGFP-fused protein- or antibody-based immuno-staining.

Although P78/83 is the viral activator for the ARP2/3 complex to initiate the actin polymerization process in the nuclei of AcMNPV-infected cells, the viral WASP homologous protein is demonstrated to be self-localized in the cytoplasm of uninfected cells and its nuclear relocation mechanism in infected cells has not yet been elucidated (5). By cotransfection assays, we identified that C42 was capable of relocating P78/83 to the nuclei in uninfected cells, and the NLS motif in C42 was demonstrated to be critical for this relocation process. To validate our preliminary results in bacmid-transfected cells, a mutant bacmid with *c42* disruption was generated by λ Red recombination and two rescued constructs containing wild-type *c42* or NLS coding sequence-mutated *c42* were also prepared by site-specific transposition. By identification with immuno-staining, the subcellular distribution pattern of P78/83 was altered in either vAc$^{c42ko\text{-}gfp}$- or vAc$^{c42nls\text{-}gfp}$-transfected cells, in which P78/83 was blocked from being imported to the nuclei, and this abnormal cytoplasmic distribution pattern presented a striking contrast to the normal nuclear distribution pattern of P78/83 in either vAc$^{c42res\text{-}gfp}$-transfected cells or AcMNPV-infected cells (6). The bacmid transfection tests not only confirmed the results from the cotransfection assays that C42 and its NLS motif were engaged in relocating P78/83 to the nuclei in uninfected cells but also extended our understanding from uninfected cells to bacmid-transfected cells. Therefore, the discovery that C42 was involved in determining the subcellular distribution pattern of P78/83 in *trans* aroused our interest to further investigate the downstream effects of abnormally distributed P78/83 caused by the *c42* knockout or mutation. We next assessed the nuclear actin polymerization activities in both vAc$^{c42ko\text{-}gfp}$- and vAc$^{c42nls\text{-}gfp}$-transfected cells. As expected, no visible F-actin cables were discerned in the nuclei of the bacmid-transfected cells by F-actin-specific staining, which could be attributed to the absence of P78/83 in the nuclei, which resulted in a failure to activate the ARP2/3 complex, which initiates the nuclear actin polymerization process. Combined with previous data that indicate that C42 binds to P78/83 in infected cells in a nucleocapsid-independent manner and that both of them share the same temporal expression pattern (1, 22), a likely scenario for the relocation of P78/83 to the nuclei in AcMNPV-infected cells is that C42 binds to P78/83 in the cytoplasm to form a viral protein complex and that this protein complex is then relocated to the nuclei under the direction of C42's NLS motif. When the NLS motif in C42 was mutated, the nuclear relocation process of P78/83 was interrupted either by the failure to receive guidance from the cytoplasm-localized C42 mutant or as a consequence of being detached from the C42 mutant if their interaction domain was destroyed by the NLS mutation.

In fact, this cotransportation mode is not rare in virus-infected cells. The AcMNPV viral protein LEF-3, which contains a nontypical NLS motif at its N-terminal region, was shown to be engaged in relocating P143, a cytoplasmic self-localization protein, to the nuclei in AcMNPV-infected cells via its residues between positions 83 and 125, which are essential for interaction with P143 in *trans* (3). A similar cotransportation mode also occurred in a cytomegalovirus infection. The cytomegalovirus assembly protein precursor contains dual NLS motifs which interact with the major capsid protein, and this interaction is required for the nuclear entry of the major capsid protein, which otherwise remains in the cytoplasm of uninfected cells.

Previous research demonstrated that viral nucleocapsid synthesis is interrupted in the presence of microfilament inhibitor cytochalasin, and no well-defined nucleocapsid structures could be discerned in the cells transfected with *c42*-disrupted bacmid (20, 23). Herein, it is not difficult to associate these two phenotypes with P78/83, as our experiments demonstrated that P78/83 was blocked from entering the nuclei and so was unable to activate the ARP2/3 complex to initiate actin polymerization in vAc$^{c42ko\text{-}gfp}$-transfected cells. The subsequent nuclear F-actin formation deficiency will lead to the interruption of viral nucleocapsid synthesis in a mechanism similar to inhibition with cytochalasin.

Finally, a previous report hypothesized that P78/83 was possibly a component of the virus-induced RNA polymerase (9). It seems that our findings did not support this idea, as qRT-PCR results demonstrated that there were no significant transcriptional depressions of viral late and very late genes in vAc^{c42ko}-transfected cells, in which P78/83 was located in the cytoplasm rather than the nuclei, where a putative RNA polymerase should be located in order to function as a viral transcriptase.

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